# Circulating immune complexes and in vitro cell reactivity in paracoccidioidomycosis

M. Arango[1], F. Oropeza[2], O. Anderson[2], C. Contreras[2], N. Bianco[2] & L. Yarzábal[1]
[1] *Sección de Inmunología Parasitaria, CEPIALET, OPS/OMS, Instituto Nacional de Dermatología, U.C.V., Apartado 4043, Caracas 1010 A and* [2] *Centro Nacional de Referencia en Inmunologia Clínica, SAS-UCV, Instituto Anatomo-Patológico, U.C.V., Caracas, Venezuela*

## Abstract

The severest forms of paracoccidioidomycosis (Pcm) are associated with impaired cell-mediated immunity, a phenomenon that is reversible with therapy. It has been postulated that plasma factors could be responsible for such immune dysfunction. In this report, circulating immune complexes (CIC) were measured by the Raji cell radioimmunoassay (Raji) and by the $^{125}$I-C1q binding assay (C1q-BA) in sera from 14 patients with either active or inactive forms of Pcm and from 15 healthy controls. The C1q-BA revealed significantly elevated levels of CIC in the sera of all but one of the patients. Four of the 8 active (62%) and 2 of the 6 inactive (33%) patients had CIC levels significantly higher than the controls as determined by the Raji test. Significantly increased levels of CIC were detected only in the active patients by the Raji test. The serum of one of the patients, with a generalized infection and depressed lymphocyte responsiveness, was examined and found to contain a factor which depressed the in vitro proliferation of both homologous and normal lymphocytes. We also found that pre-culture of the patients' lymphocytes before stimulation restored their proliferative capacity, and IC were detectable in the culture supernatants. However, the subsequent addition of the patients' serum to such precultured cells did not reinduce the depression. It is suggested therefore, that the depression of T cell responses observed in Pcm is due to the presence of IC which may interact reversibly with the responding cells and/or activate a suppressor cell population whose activity is diminished by preculture.

## Introduction

In recent years evidence has been accumulated which indicates that progressive disseminated forms of paracoccidioidomycosis (Pcm) are associated with various degrees of depressed cell - mediated immunity. A number of workers have demonstrated the existence of a non specific T-cell hyporeactivity using both in vivo and in vitro methods, in this fungal infection (1, 2, 3, 6). Apparently this is an acquired phenomenon since T-cell function is partially or completely restored after succesful therapy (1, 6). It has been postulated that soluble serum factors (alpha globulins, antibodies, antigens, immune complexes) could be responsible for such immune dysfunction (3, 6), but none of these substances have been definitively identified as the inhibitory agent. Musatti *et al.* (3) did not find a correlation between serum antibody levels and T-cell hyporeactivity, and Restrepo *et al.* (6) failed to demonstrate a correlation between alpha 2 microglobulin levels and impairment of cellular immunity. Studies were therefore undertaken in our laboratories to investigate the presence of circulating immune complexes in well documented cases of Pcm, and to examine serum suppressor activity in those patients suffering from disease-related immunosuppression.

Mycopathologia 79, 153-158 (1982). 0301-486x/82/0793-0153/$1.20.

## Materials and methods

### *Patient population*

The 14 patients with Pcm, mycologically confirmed by culture, were part of a larger group reported in detail elsewhere (1). They were divided into two groups on the basis of clinical evaluation at the time of study (Table 1). Group 1 contained 8 patients with active, positive culture, nontreated Pcm. Three of these had generalized depression of cell-mediated immunity, four showed varied degrees of T-cell hyporeactivity, and one exhibited good T-cell responses. Group 2 was comprised of 6 patients with inactive, negative culture, sulphonamide treated Pcm. All had appreciable levels of T-cell mediated immunity. Group 3 was comprised of 15 healthy subjects living in endemic rural areas of Venezuela and were comparable to the patients in their socio-economic level and racial origin.

### *$^{125}$I-C1q binding test (C1q-BA)*

The sera were tested as described by Zubler *et al.* (9), with minor modifications. In brief, 50 $\mu$l of test serum was incubated with 100 $\mu$l of EDTA (0.2 M, pH 8.3) for 30 min at 37 °C. After this 50 $\mu$l of $^{125}$-C1q and 1 ml of 3% polyethylene glycol (PEG) in borate buffer (pH 8.3) were added and following incubation at 4 °C for 4 hr, the tubes were centrifuged at 1.500 × g for 20 min at 4 °C. The supernatant was then decanted and the residual radioactivity was counted without washing the pellet. The results were expressed as the percentage of radioactivity precipitated compared to tubes in which 1 ml of 20% trichloroacetic acid was added as the precipitant. The percentage of the protein bound radioactivity which was included in the precipitate was referred to as the 'C1q binding activity' of the sample.

*Table 1.* Clinical and immunological status of patients with active (group 1) or inactive (group 2) paracoccidioidomycosis.

| Subjects | Age (years) Sex | Cutaneous reactivity | | | | Lymphocyte transformation | | | Serum ES (N° bands) | Antibodies Micro-Elisa ($log_2$ titres) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | DNCB | PPD (mm) | Ca (mm) | Pbs (mm) | PHA (si) | Ca (si) | Pb (si) | | |
| **Group 1** | | | | | | | | | | |
| IB | 47/M | + | 0 | 10 | 0 | 40.0 | 1.5 | 1.0 | 6 | 10.64 |
| VB | 54/M | – | 0 | 0 | 0 | 3.0 | 1.0 | 1.0 | 9 | 11.64 |
| LE | 51/F | + | 13 | 12 | 11 | 48.0 | 7.0 | 27.5 | 8 | 9.64 |
| FI | 35/M | + | 0 | 9 | 10 | 60.0 | 2.0 | 10.0 | 4 | 8.64 |
| AM | 16/M | – | 0 | 0 | 0 | 11.8 | 1.0 | 1.0 | 9 | 13.64 |
| JM | 27/M | – | 0 | 0 | 8 | 28.0 | 2.2 | 6.3 | 4 | 10.64 |
| RP | 38/M | + | 0 | 5 | 0 | 40.0 | 1.8 | 1.2 | 7 | 12.64 |
| JS | 51/M | – | 8 | 12 | 10 | 35.0 | 8.2 | 6.7 | 6 | 13.64 |
| **Group 2** | | | | | | | | | | |
| AA | 65/M | + | 7 | 8 | 5 | 70.0 | 13.7 | 18.0 | 1 | 7.64 |
| WN | 10/M | + | 13 | 10 | 0 | 76.5 | 7.6 | 15.1 | 3 | 7.64 |
| AP | 57/M | + | 16 | 0 | 14 | 67.7 | 6.1 | 28.0 | 3 | 8.64 |
| CP | 51/M | + | 8 | 6 | 10 | 70.0 | 3.7 | 25.0 | ND | ND |
| MR | 41/M | – | 13 | 20 | 12 | 113.0 | 13.5 | 9.4 | 0 | 8.64 |
| RT | 24/M | – | 15 | 7 | 15 | 117.0 | 3.4 | 43.0 | 5 | 8.64 |

DNCB = 2,4-dinitrochlorobenzene
PPD = *Mycobacterium tuberculosis* purified protein derivative
Ca = Candidin
Pbs = Paracoccidioidin
PHA = Phytohemaglutinin
Pb = Non viable particulate *Paracoccidioides brasiliensis* antigen
ES = Electrosyneresis
ELISA = Enzyme-linked-immunosorbent assay
SI = Stimulation Index

*Raji cell radioimmunoassay*

This technique was performed as described by Theofilopoulos *et al.* (7). In brief, $2 \times 10^6$ Raji cells suspended in 50 $\mu$l Hank's balanced salt solution (BSS) were mixed with 25 $\mu$l 1:4 diluted test serum, incubated at 37 °C for 45 min, and washed 3 times with Hank's BSS. The washed cells were allowed to react at 4 °C for 30 min with an optimal amount of $^{125}$I-rabbit antihuman IgG. After incubation, the cells were washed three times and the uptake of radioactivity was determined in a gamma counter. The binding of the tests was compared with the standard curve obtained using Raji cells incubated with increasing concentrations of aggregated human gamma globulin (AHG) in normal human serum. The results were expressed as AHG equivalents per ml.

The sera were tested in duplicate after a single thawing, for both the C1q-BA and the Raji test.

*Influence of the serum of a Pcm patient (AM) on lymphocyte function*

Experiments were designed to explore the effect of the serum of an active Pcm patient, who had disease-related immunedepression, on lymphocyte proliferation. Enriched T lymphocytes were prepared from the peripheral blood of normal individuals or Pcm patients (1) by sedimentation of the erythrocytes and removal of glass-adherent cells. They were stimulated with optimal concentrations of PHA, *P. brasiliensis* or *C. albicans* in the presence of autologous or AM serum.

*Influence of pre-culture upon the reactivity of depressed lymphocytes*

Peripheral mononuclear cells of AM were purified by the procedure described above. A portion of these cells was stimulated directly with optimal concentrations of PHA or *P. brasiliensis*. A different aliquot was washed and then stimulated in the presence of autologous, or homologous normal serum. The stimuli used were PHA, and when appropriate, *P. brasiliensis* or *C. albicans* antigens. Parallel aliquots of washed cells were pre-incubated for 18 hr at 37 °C in McCoy medium then stimulated either in the presence of autologous, homologous or fetal calf serum.

*Statistical analysis*

The statistical comparison of the CIC levels of the three study groups was performed with the Mann-Whitney U test.

## Results

*Levels of immune complexes*

As can be seen in Table 2, the C1q-binding activity (C1q-BA) of the normal sera included in the study was always less than 3.2% (range 0.85 to 3.16), and the highest value generated by the Raji

*Table 2.* Results of the C1q binding and the Raji cell tests in sera from paracoccidioidomycosis patients and normal individuals.

| Patients | C1q binding test % binding | Raji cell technique $\mu$g AHGG equivalents |
|---|---|---|
| **Group 1** | | |
| IB | 21.50 | 118.75 |
| LE | 20.80 | 30.00 |
| JM | 14.00 | 6.25 |
| JS | 11.91 | 75.00 |
| VB | 11.50 | 675.00 |
| RP | 9.10 | 6.25 |
| FI | 7.20 | 6.25 |
| AM | 5.50 | 1000.00 |
| **Group 2** | | |
| CP | 87.00 | 37.48 |
| AP | 44.80 | 19.50 |
| AA | 23.20 | 37.48 |
| RT | 20.40 | 6.25 |
| WN | 14.80 | 6.25 |
| MR | 1.90 | 6.25 |
| **Group 3** | | |
| AV | 3.16 | 11.87 |
| MC | 3.06 | 4.70 |
| RV | 3.05 | 19.70 |
| ZR | 2.97 | 4.99 |
| MB | 2.86 | 3.15 |
| IR | 2.77 | 34.40 |
| MM | 2.48 | 3.93 |
| IB | 2.29 | 4.70 |
| TP | 2.09 | 13.70 |
| HC | 1.93 | 15.20 |
| NM | 1.70 | 3.77 |
| SR | 1.24 | 4.12 |
| CV | 1.10 | 26.60 |
| ES | 1.03 | 3.15 |
| DM | 0.85 | 4.20 |

cell technique in the same group of sera was 34.40 μg equivalents of AHG/ml (range 3.15 to 34.40). According to these values, the C1q-BA revealed abnormally elevated levels of CIC in all the active patients and in 5 of the 6 inactive subjects. Differences observed between active or inactive patients and controls were statistically significant ($p < 0.001$ and 0.01 respectively). In contrast, the Raji cell technique detected significantly elevated values of CIC in only 4 of the 8 active patients and in 2 of the 6 inactive individuals. Differences detected between active patients and controls were statistically significant ($p < 0.05$).

The highest levels detected by the Raji cell assay were observed in the patients VB and AM (Table 2) who showed marked depression of T-cell mediated immunity (Table 1). This inverse correlation was not seen wen the C1q-BA test was employed.

*Influence of AM serum on normal lymphocyte function*

As can be seen in Table 3, the serum of patient AM exerted an inhibitory influence on the in vitro reactivity of normal lymphocytes from 3 healthy donors when stimulated by PHA. A similar effect was observed when lymphocytes from 2 healthy donors, sensitized to *P. brasiliensis* or *C. albicans*, were stimulated with the non-viable particulate or soluble antigens of the fungi in the presence of AM serum.

*Influence of pre-culture upon the reactivity of depressed lymphocytes*

As can be seen in Table 4, the proliferative capacity of AM's lymphocytes depended to a substantial degree upon the conditions of culture. His lymphocytes, when stimulated directly, exhibited little response, but after washing and stimulation in either homologous or heterologous serum, significant responses to PHA were observed. After pre-incubation for 18 hr, the responses approached those of normal individuals when stimulated either in the presence of autologous or heterologous serum. The Raji test detected 37.5 μg equivalents of AGH/ml in the preculture supernatant.

*Evolution of lymphocyte reactivity and CIC levels in patient AM*

The evolution of the immune responsiveness of patient AM during treatment is presented in Figure 1. As can be seen, during 18 months treatment with sulphonamides and amphotericin B, LT, responses to *P. brasiliensis* that were absent in the first few months, gradually became equivalent to those in responsive patients. The level of CIC as measured by the Raji test behaved inversely, gradually falling from 2600 μg to 3.5 μg equivalents of AHG/ml, resembling those of normal individuals. Anti *P. brasiliensis* antibody titres, measured by micro-ELISA (1), which were originally very high also pro-

*Table 3.* Proliferation of lymphocytes of reactive individuals in Autologous Serum (AS) and that of patient AM (AMS).

| Lymphocyte donor | Stimulation index | | | | | |
|---|---|---|---|---|---|---|
| | PHA | | C. albicans | | P. brasiliensis | |
| | AS | AMS | AS | AMS | AS | AMS |
| BP | 76.51 | 24.05 | | | | |
| AH | 112.20 | 1.20 | | | | |
| DV | 22.00 | 6.26 | | | | |
| EM | | | 5.00 | 1.20 | | |
| IG | | | 4.20 | 1.00 | | |
| FH | | | | | 27.78 | 1.15 |
| ER | | | | | 99.00 | 3.33 |

PHA = Phytohemaglutinin

*Table 4.* Influence of different treatments on the in vitro proliferative response of AM lymphocytes.

| Treatment | Stimulation Index | |
|---|---|---|
| | PHA | *P. brasiliensis* |
| Stimulated directly | 3.92 | 1.0 |
| Washed then stimulated in: | | |
| autologous serum | 24.00 | 1.0 |
| homologous serum | 30.15 | 1.7 |
| Washed then incubated 18 hrs in medium containing homologous serum and stimulated in: | | |
| autologous serum | 93.00 | ND |
| fetal calf serum | 100.77 | ND |
| homologous serum | 151.00 | 5.8 |

PHA = Phytohemaglutinin
ND = Not done

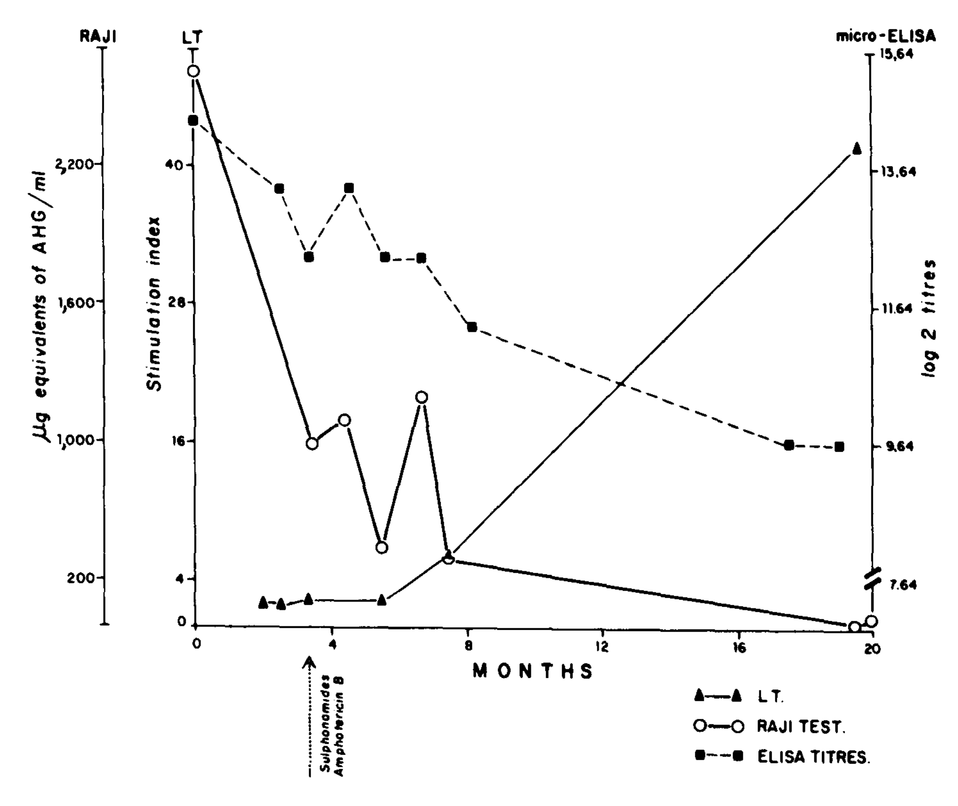



*Fig. 1.* Evolution of cellular and humoral immunity during treatment of a Pcm patient (AM) with disease-related immunodepression.

gressively fell from 1:25 000 to 1:800. Total IgE levels fluctuated considerably, but showed no consistent trend during the period of study.

## Discussion

Considerable evidence exists to suggest that cell mediated immunity (CMI) has a primary role in host defense against *P. brasiliensis* infection. Thus it has been observed that persons who have recovered definitively from this infection have delayed cutaneous reactivity against fungal antigens, and in vitro lymphocyte proliferation occurs upon exposure to such materials (3). In contrast, persons with the progressive disseminated form of the disease demonstrate diverse grades of depression of CMI (1, 2, 3, 6). In this later group, the alterations in lymphocyte function tend to normalize after successful therapy of the patients (1, 6). In addition, in the severe forms of the disease, high titres of specific antibodies are detected, thus indicating that these have little protective effect in Pcm (1, 6). It appears that the diminished T cell reactivity in Pcm is not due to lowered T cell numbers, but rather to functional alterations in this population.

The first important finding of the present study is the detection of CIC in Pcm patients. These were found in the sera of both patients with active or inactive infections when the Clq binding test was used, and predominantly in the active cases by the Raji test. The lack of correlation between these two tests might possibly be explained by their detection of complexes of distinct molecular weights and/or chemical composition. Both of these possibilities require further study to be definitively verified.

In a recent publication (8) a high proportion of patients suffering active coccidioidomycosis were found to possess CIC in concentrations that were significantly greater than in inactive or control, subjects. These authors also emphasized the variable results obtained by the application of different CIC assay systems, and demonstrated the presence of coccidioidin antigen in isolated complexes.

The second fundamental observation arising from this work is the apparent association between CIC levels, determined by the Raji test, and depressed T cell responsiveness. This relation can be

postulated because the highest levels of CIC detected by this method were in two anergic patients who had markedly depressed lymphocyte reactivity in vitro.

Furthermore, in one of these patients who was studied longitudinally, recovery of lymphocyte responsiveness was accompanied by a diminution of CIC levels during treatment. Thus, CIC may inhibit T cell function, either directly at the level of the effector cells, or by stimulating suppressor subpopulations. Our experiments designed to study the effects of culture on lymphocyte reactivity in vitro, revealed that pre-culture of anergic lymphocytes for 18 hrs. at 37 °C was capable of allowing the re-expression of the specific stimulatory effects of *P. brasiliensis* or other antigens, and also non-specific mitogen reactivity. This effect was observed whether the stimulation was performed in autologous, heterologous or fetal calf serum. IC were also detected in the pre-culture supernatant. It is important to note that the serum of the anergic patient also inhibited the in vitro proliferative response of normal lymphocytes to mitogens and antigens. This inhibitory activity of the patients' serum may be due to CIC blocking lymphocytes surface receptors, thus interfering with their function. The reversal of this inhibition of function of the patients lymphocytes by pre-culture may be due to shedding of the CIC. This mechanism has in fact been invoked to explain the recovery of depressed lymphocyte function in systemic lupus erythematous (9) and other diseases.

However, our results indicate that this may not be the only change occurring during pre-culture. Thus, the addition of the patients' serum to pre-cultured lymphocytes did not exert the inhibitory effect that was observed when non-precultured cells were used. This suggests that the proliferative stimulus might arrive before the CIC exert their suppressive effect. On the basis of these data we postulate, and plan to further investigate, the possibility that CIC are in fact activating a suppresor population, and that this population may lose its surface receptors during the period of pre-culture.

In summary, our finding support the assumption that the depression of the T-cell mediated immunity, observed in progressive disseminated forms of paracoccidioidomycosis, may not result from a lack of potentially reactive T-cells, but rather from suppression of this reactivity by blocking of effector cell surface receptors and/or by the action of suppressor cells. A comparable mechanism has recently been suggested to operate in onchocerciasis (4).

## Acknowledgements

This study was supported in part by the Venezuelan National Council for Science and Technology (CONICIT), Grant N° DDCT-Sal. 2. We wish to thank Doctors M. C. B. Albornoz, H. Campins and C. L. Herrera for referring patients; and Doctors A. Restrepo and N. Lynch for valuable discussions and for assistance in preparing this manuscript. We are indebted to the 34 persons who volunteered for this study.

## References

1. Arango, M. & Yarbázal, L. A., 1982. T.cell dysfunction and hyperimmuno glubulinemia E in paracoccidioidomycosis. Mycopathologia 79: 115 123.
2. Mok, P. W. Y. & Greer, D. L., 1977. Cell-mediated immune response in patients with paracoccidioidomycosis. Clin. Exp. Immunol. 28: 89–98.
3. Musatti, C. C., Rezkallah, M. T., Mendes, E. & Mendes, N. F., 1976. In vivo and in vitro evaluation of cell-mediated immunity in patients with paracoccidioidomycosis. Cell. Immun. 24: 365–378.
4. Paganelli, R., Ngu, J. L. & Levinsky, R. J., 1980. Circulating immune complexes in onchocerciasis. Clin. Exp. Immunol., 39: 570–575.
5. Pérez-Rojas, G., Suárez-Chacón, R., Penchaszadeh, G., Tapanes, F., Contreras, C. E., Abadi, I., Pinto, A., Armas, P., Zschaeck, D., Boissiere, M. & Bianco, N. Pathophysiology of the immune response in systemic lupus erythematosus: a new approach. In: Streptococcal disease and the immune response. Ed. by S. E. Read & J. B. Zabriskie, Academic Press, pp. 507.
6. Restrepo, A., Restrepo, M., Restrepo, F., Aristizabal, L. H., Moncada, L. H. & Velez, H., 1978. Immune responses in paracoccidioidomycosis. A controlled study of 16 patients before and after treatment. Sabouraudia. 16: 151–163.
7. Theofilopoulos, A. N., Wilson, C. B. & Dixon, F. J., 1976. The Raji Cell radioimmune assay for detecting immune complexes in human sera. J. Clin. Inv. 57: 169–182.
8. Yoshinoya, S., Cox, R. E. & Pope, R. M., 1980. Circulating immune complexes in coccidioidomycosis. Detection and characterization. J. Clin. Inv. 66: 655–663.
9. Zubler, R. H., Lange, G., Lambert, P. H. & Miescher, P. A., 1976. Detection of immune complexes in unheated sera by a modified $^{125}$I-C1q binding test. Effect of heating on the binding of C1q by immune complexes and application of the test to systemic lupus erythematosus. J. Immunol. 116: 231–138.